User Manual

pEcoli Expression Systems User Manual

Clontech

United States/Canada
800.662.2566
Asia Pacific
+1.650.919.7300
Europe
+33.(0)1.3904.6880
Japan
+81.(0)77.543.6116

Clontech Laboratories, Inc.
A Takara Bio Company
1290 Terra Bella Ave.
Mountain View, CA 94043
Technical Support (US)
E-mail: tech@clontech.com
www.clontech.com

Cat. Nos. 631417 & 631418
PT5018-1 (012512)

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Customer Service/Ordering
tel: 800.662.2566 (toll-free)
fax: 800.424.1350 (toll-free)
web: www.clontech.com
e-mail: orders@clontech.com

Technical Support
tel: 800.662.2566 (toll-free)
fax: 650.424.1064
e-mail: tech@clontech.com

I. Introduction & Protocol Overview

The **pEcoli Expression System** and **pEcoli Linear Expression System** are designed for the cloning and expression of his-tagged proteins using *E. coli* (Figure 1). Bacterial expression is typically the first approach to the production of proteins for *in vitro* studies, due to its simplicity and robustness. Our Bacterial Expression Systems have been designed to expedite the use of bacterial expression by incorporating a number of technologies that increase the efficiency of each of these steps. This system, which is based on the inducible T7 expression system (pET) developed by F. William Studier and colleagues at Brookhaven National Laboratories (Moffatt & Studier, 1986, Rosenberg *et al.*, 1987, Studier *et al.*, 1990), contains IPTG-inducible, pET-based vectors providing high levels of protein expression.

Choice of cloning options

The pEcoli Expression System (Cat. No. 631417) contains two separate bacterial expression vectors encoding N- or C-terminal 6xHN fusion tags (pEcoli-Nterm-6xHN and pEcoli-Cterm-6xHN, respectively), which provide a variety of cloning options (see Figure 2). These vectors, which are circular, contain multiple cloning sites and can be used for traditional restriction enzyme cloning. The pEcoli Expression System is available separately, or as part of the EP ("**E**xpress and **P**urify") Starter Kits. The pEcoli Linear Expression System (Cat. No. 631418) contains prelinearized versions of these vectors, utilizing our In-Fusion® technology to provide seamless (no addition of base pairs), easy, low-background directional cloning of PCR products, without the need for restriction enzyme digestion, subcloning, or *in vitro* ligation. The pEcoli Linear Expression System is available separately, or as part of the CEP ("**C**lone, **E**xpress and **P**urify") Starter Kits. See Sections V and VI, respectively, for details regarding these two cloning methods.

Tightly regulated expression

The vectors supplied with these systems contain the hybrid T7 *lac* promoter, as well as a *lacI* gene, which encodes Lac repressor (Dubendorff & Studier, 1991). This combination reduces expression in the absence of inducer, while allowing for rapid inducibility upon addition of IPTG to the bacterial culture (see Figure 3). This reduces background expression levels, since Lac repressor binds to the *lac* operator present adjacent to the T7 promoter.

Easy purification

The provided vectors are designed to incorporate 6xHN tags at the amino- or carboxy-terminus of the protein of interest, allowing for convenient purification using Immobilized Metal Ion Affinity Chromatography (IMAC) (Figure 4). The 6xHN tag is short enough not to interfere with protein biological activity and function, and long enough not to be internalized.

The **EP Starter Kits**, which contain the pEcoli Expression System, and the **CEP Starter Kits**, which contain the pEcoli Linear Expression System, come with your choice of purification resin and buffers to perform extraction and purification of his-tagged proteins. Choose HisTALON™ Co resin to achieve the highest purity of your target protein—or choose His60 Ni Superflow resin when a high binding capacity is required.

I. Introduction & Protocol Overview continued

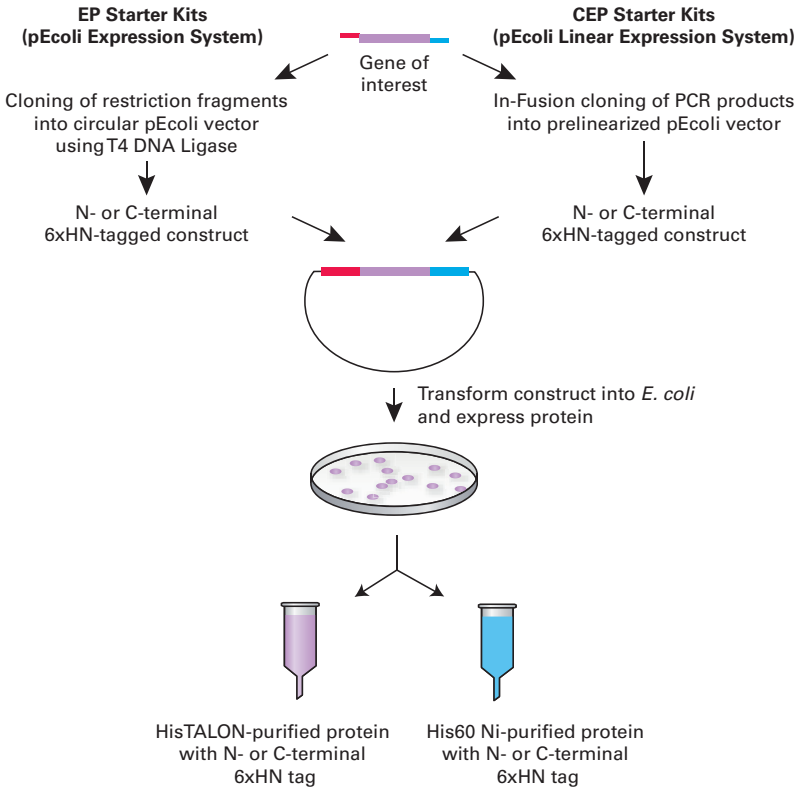

Figure 1. The EP and CEP Starter Kits protocol. A gene of interest is cloned into a pEcoli vector, a pET-based expression system, to generate an N- or C-terminal-tagged construct. The pEcoli Expression System allows a choice of cloning sites, while the pEcoli Linear Expression System provides the option of easy, precise In-Fusion PCR cloning to yield a 6xHN-tagged construct. After transformation and growth in *E. coli*, the expressed N- or C-terminal-tagged protein is efficiently purified using either HisTALON resin for highest purity of the target protein or His60 Ni Superflow resin for the highest binding capacity.

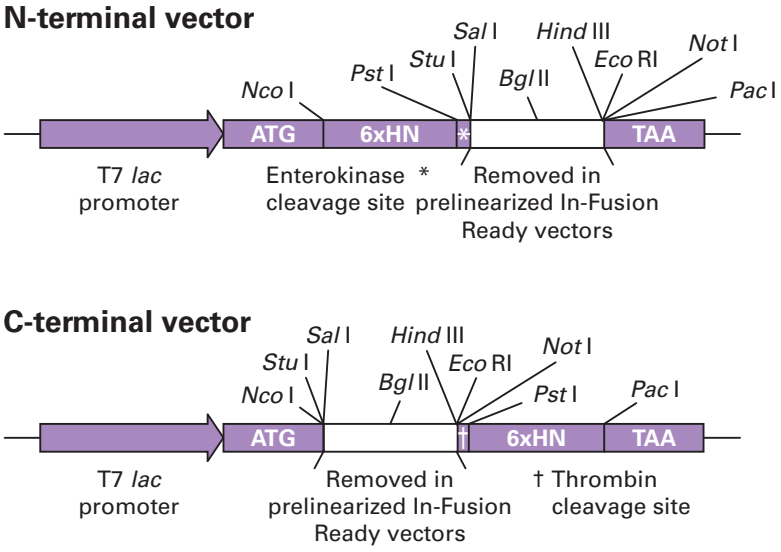

Figure 2. Cloning options using the pEcoli Expression System. Features of the multiple cloning sites of our pEcoli Expression Vectors are shown (not to scale). Transcription proceeds from left to right, beginning at ATG and ending at the stop (TAA) codon shown. Convenient restriction sites (indicated) allow inclusion or removal of tags as needed. The unshaded region is removed in our pEcoli Linear Expression Vectors using Sal I and Hind III restriction enzymes.

I. Introduction & Protocol Overview continued

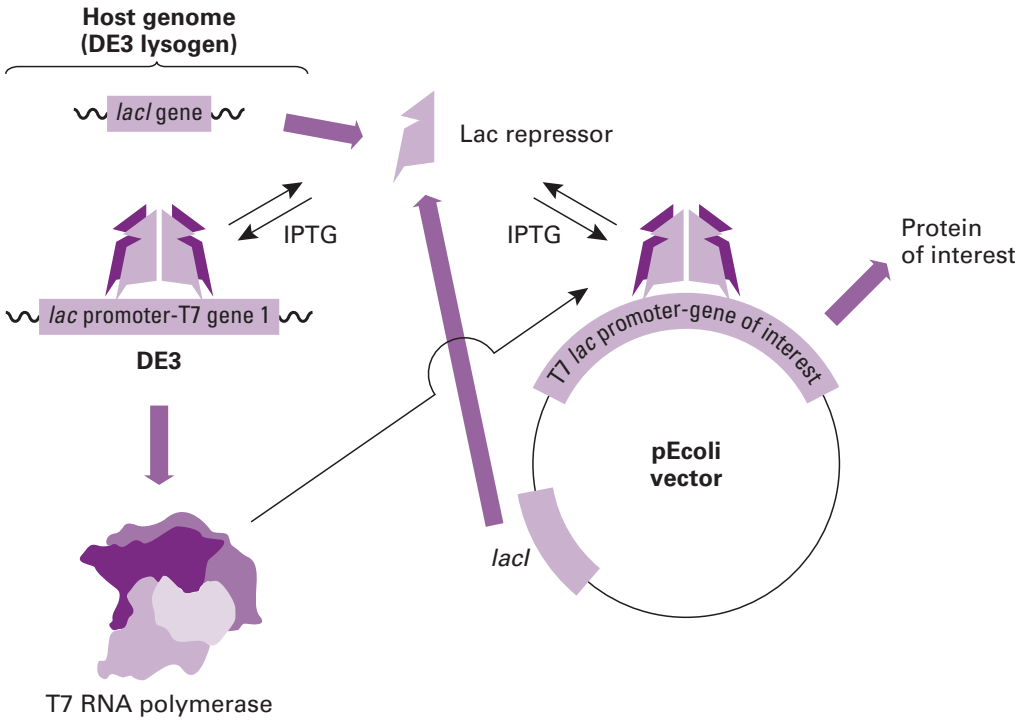

Figure 3. The molecular basis of the pET system for recombinant protein expression in DE3 lysogens. Tight regulation is achieved by the presence of *lac* operator sites in two promoters: the *lac* UV5 promoter that controls expression of T7 RNA polymerase (integrated into the genome of the DE3 lysogen) and the hybrid T7 *lac* promoter that controls expression of the gene of interest. In the absence of inducer (IPTG), Lac repressor (expressed from both genomic and plasmid-derived *lacI* genes) binds tightly to the operator sites to repress transcription. When IPTG is added during induction, Lac repressor releases from the operators, allowing expression of T7 RNA polymerase, which then acts on the newly derepressed T7 *lac* promoter. The thick arrows refer to expression of gene products, while the thin arrows indicate molecular interactions.

II. List of Components

Always store any unused In-Fusion Dry-Down Mix in a desiccator at 20–22°C.
Store all other components at –20°C.

pEcoli Expression System (Cat. No. 631417)

- 10 µg pEcoli-Nterm-6xHN Vector (500 ng/µl)
- 10 µg pEcoli-Cterm-6xHN Vector (500 ng/µl)
- 5 µg pEcoli-6xHN-GFPuv Vector (500 ng/µl)

- pEcoli Expression Systems User Manual (PT5018-1)
- pEcoli-Nterm-6xHN Vector Information Packet (PT3868-5)
- pEcoli-Cterm-6xHN Vector Information Packet (PT3869-5)
- pEcoli-6xHN-GFPuv Vector Information Packet (PT3870-5)

pEcoli Linear Expression System (Cat. No. 631418)

- 8 rxns In-Fusion Dry-Down Mix (1 x 8-well strip)
- 1.5 µg pEcoli-Nterm-6xHN Linear Vector (100 ng/µl)
- 1.5 µg pEcoli-Cterm-6xHN Linear Vector (100 ng/µl)
- 5 µg pEcoli-6xHN-GFPuv Vector (500 ng/µl)
- 5 µl 1.1 kb *LacZ*-RK Control Insert (25 ng/µl)

- pEcoli Expression Systems User Manual (PT5018-1)
- pEcoli-Nterm 6xHN Linear (In-Fusion Ready) Vector Information Packet (PT3871-5)
- pEcoli-Cterm 6xHN Linear (In-Fusion Ready) Vector Information Packet (PT3872-5)
- pEcoli-6xHN-GFPuv Vector Information Packet (PT3870-5)

III. Additional Materials Required

The following materials are required but not supplied:

- **Advantage® HD Polymerase Mix** (Cat. Nos. 639241) for amplifying your gene of interest
- **NucleoSpin Gel and PCR Clean-Up** (Cat. No. 740609.50) for purification of vector and insert (if using pEcoli Expression System), or purification of insert (if using pEcoli Linear Expression System)
- **DNA Ligation Kit, Ver 1.0** (TAKARA Bio USA, Cat. No. TAK 6021) or **DNA Ligation Kit, Ver 2.1** (TAKARA Bio USA, Cat. No. TAK 6022)
- **High Fidelity PCR EcoDry™ Premix** (Cat. No. 639280) for colony PCR screening
- **NucleoSpin QuickPure** (Cat. No. 740615.50) or **NucleoSpin Plasmid** (Cat. No. 740588.50) for purification of positive clones following colony screening
- **TALON® xTractor Buffer** (Cat. No. 635656) for solubility analysis and extraction of expressed proteins
- **ProteoGuard EDTA-Free Protease Inhibitor Cocktail** (Cat. Nos. 635672 or 635673)
- **5X SDS loading buffer**
  - 15% β-mercaptoethanol
  - 15% SDS
  - 50% glycerol
  - 1.5% bromophenol blue
- **Stellar™ Electrocompetent Cells** (Cat. No. 636765)
- **BL21 (DE3) competent bacteria** (EMD Biosciences, Inc.)
- **ampicillin** (100 mg/ml stock)
- **LB (Luria-Bertani) medium (pH 7.0)**
  for 1 L
  - 1.0% Bacto-tryptone (10 g)
  - 0.5% yeast extract (5 g)
  - 1.0% NaCl (10 g)

  Dissolve ingredients in 950 ml of deionized H_2O. Adjust the pH to 7.0 with 5 M NaOH and bring the volume up to 1 L. Autoclave on liquid cycle for 20 min at 15 lb/in^2. Store at room temperature or at 4°C.
- **LB/antibiotic plates**
  Prepare LB medium as above, but add 15 g/L of agar before autoclaving. Autoclave on liquid cycle for 20 min at 15 lb/in^2. Let cool to ~55°C, add antibiotic (e.g., 100 µg/ml of ampicillin), and pour into 10 cm plates. After the plates harden, then invert and store at 4°C. For LB/X-Gal/IPTG plates, spread 40 µl each of X-Gal and IPTG stock solutions on an LB plate.
- **X-Gal stock solution** (5-bromo-4-chloro-3-indolyl-β-D-galactoside; 40 mg/ml in dimethylformamide)
  Dissolve 400 mg of X-Gal in 10 ml of dimethylformamide. Protect from light by storing in a brown bottle at –20°C.
- **IPTG stock solution** (isopropyl-β-D-thiogalactoside; 100 mM)
  Dissolve 238 mg of IPTG in 10 ml of deionized H_2O. Filter-sterilize and store in 1 ml aliquots at –20°C.

IV. Workflow

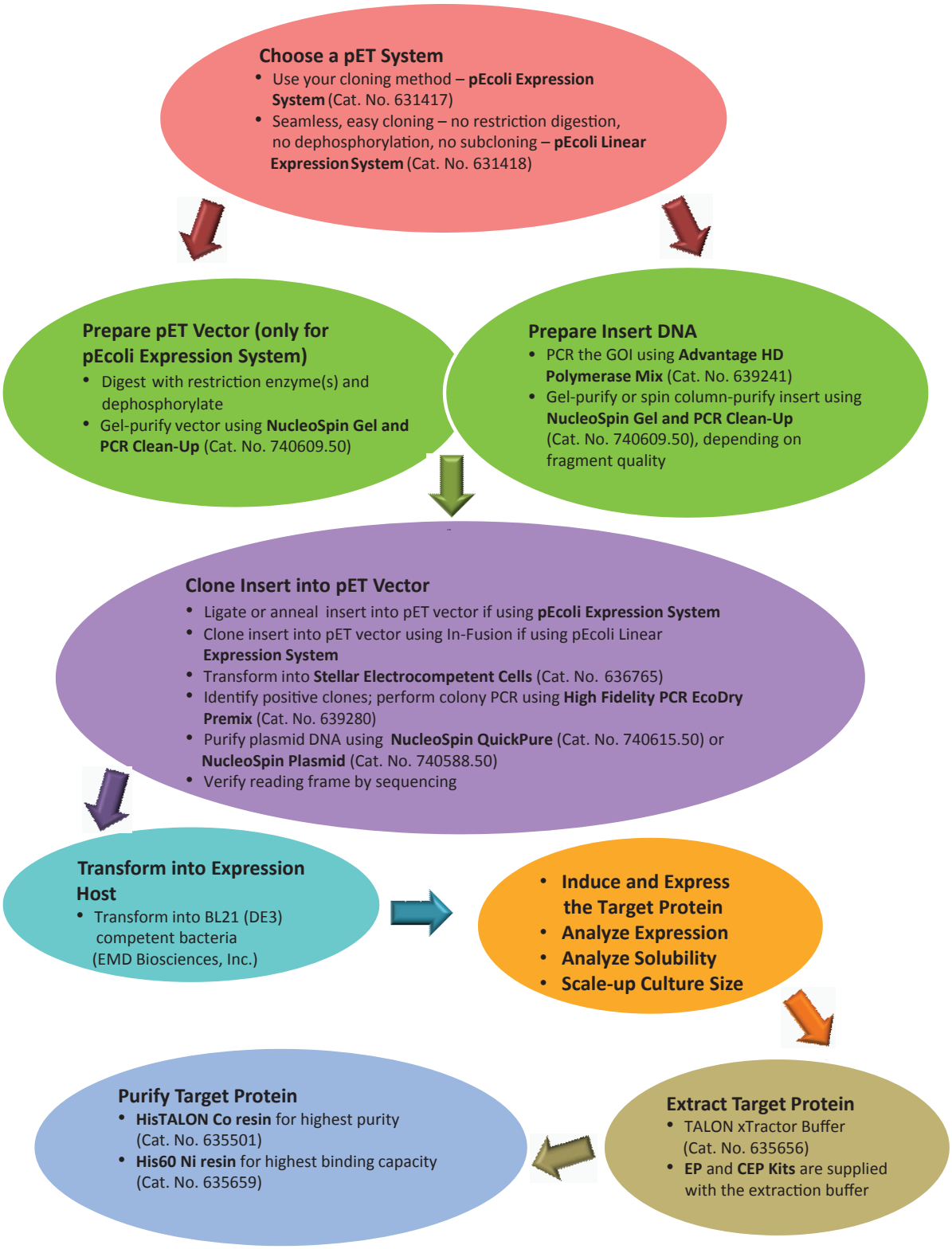

Figure 4. Bacterial expression and purification flowchart.

V. General Considerations

Traditional Cloning Method

Choose the pEcoli Expression System (Cat. No. 631417) or any of the EP Starter Kits (Cat. Nos. 631419, 631420, 631421, 631416 & 631426), which contain circular vectors for traditional restriction enzyme-based cloning. Using traditional cloning methods (i.e., ligation-based cloning of compatible DNA fragments) provides a choice of several cloning sites (Figure 2), provided that these sites are not found internally in the DNA fragment to be cloned. Traditional cloning may be more convenient if the fragment to be cloned contains restriction sites in frame with the gene of interest. For detailed vector maps, please see the Vector Information Packets listed in Section III.

PCR Cloning Method

Choose the pEcoli Linear Expression System (Cat. No. 631418) or any of the CEP Starter Kits (Cat. Nos. 631422, 631423, 631424, 631425 & 631427) which contain prelinearized vectors designed to be used with Clontech's In-Fusion technology.

In-Fusion cloning provides several advantages over restriction enzyme-based cloning:

- **Directional cloning** of PCR products into linearized vectors without the need for restriction digests, ligation, or blunt end polishing. This is more convenient than restriction enzyme-based cloning, and allows the use of restriction sites that are present within the fragment to be cloned.
- **No restriction digestion.** Since the vectors are provided in prelinearized form (using Sal I and Hind III), there is no need to digest the vector prior to cloning. In-Fusion cloning is based on homology between the ends of the linearized vector and the PCR product—this homology is introduced into the PCR product via the use of PCR primers with 5' extensions.
- **Clone the same PCR product into both vectors** in parallel. The pEcoli-Nterm 6xHN and pEcoli-Cterm 6xHN vectors have been designed so that the sequences 5' to the Sal I site and 3' to the Hind III site are the same in both vectors. This allows cloning of the same PCR product into both vectors in parallel, which is convenient for comparing expression and purification of your protein when a 6xHN tag is attached to either the amino or the carboxy terminus, respectively. Furthermore, these PCR products are compatible with all of our prelinearized fluorescent protein vectors, allowing you to easily switch to a different expression system, depending on your needs.

Vector Maps

The vector maps and multiple cloning site information for each of the pEcoli system vectors are provided in the Vector Information Packets listed in Section II, which are available at **www.clontech.com**

VI. Traditional Cloning Protocol

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING

Traditional cloning consists of many steps. These include primer design, PCR amplification, and cleanup of the PCR insert; or cleavage from an existing vector and insert cleanup. The vector must also be cleaved and cleaned up. This is followed by T4 DNA ligation, transformation, and screening of inserts. We recommend the DNA Ligation Kit, Ver. 1.0 (TAKARA Bio USA, Cat. No. TAK 6021) or the DNA Ligation Kit, Ver. 2.1 (TAKARA Bio USA, Cat. No. TAK 6022).

Protocol

A. PROTOCOL: Cloning Site Selection and PCR Primer Design

Use the following criteria to select restriction sites for cloning and to design PCR primers for generating the DNA insert:

- Choose appropriate restriction sites based on the absence of these sites within the DNA insert to be cloned.
- These sites should be incorporated into PCR primers which are then used to PCR-amplify the sequence to be cloned.
- These primers should be carefully designed to ensure that the reading frame of your gene aligns with the ATG start codon which forms part of the Nco I site at the 5' end of the MCS.
- Make certain to design your 5' primer **WITHOUT** the ATG start codon of the wild type protein.

VI. Traditional Cloning Protocol continued

- Sufficient flanking sequence should be incorporated to ensure efficient digestion of the PCR product using the appropriate restriction enzymes. We recommend six nucleotides 5' to the restriction enzyme recognition sequence. For more information on restriction enzymes, please visit TAKARA Bio USA at **www.takarabioUSA.com/am/products/subcategory/2/35**

NOTES:

- The MCS is designed with overlapping Pac I sites at the 3' end. This ensures that all three reading frames contain a stop codon. If the Pac I site is used for cloning, only one of the sites in the vector will be cut. Thus, be sure that your intended stop codon is found in the first Pac I site. That way, the stop codon will be in frame regardless of which Pac I site in the vector is digested.
- The Xba I site is 3' to the Pac I sites, and is therefore not followed by stop codons. If you use the Xba I site for cloning, be sure that your insert contains its own stop codon.

B. PROTOCOL: Generating a Gene-Specific Expression Vector

Generate your recombinant expression construct using standard molecular biology techniques, as described below. For more detailed information, see Molecular Cloning: A Laboratory Manual (Sambrook & Russell, 2001) or Current Protocols in Molecular Biology (Ausubel. *et al.*, 2001).

1. **Digest** the pEcoli vector with the restriction enzyme(s) appropriate for your expression application, treat with phosphatase (if desired), and purify.

NOTES:

- The ends of the fragment to be cloned must be compatible with one or more restriction sites present in the cloning vector. This can be accomplished by PCR-amplifying the fragment using primers that contain suitable restriction sites.
- If cloning with the 6xHN affinity tag included in the vector, ensure that the correct reading frame is maintained (see the MCS of the appropriate vector for the alignment of restriction sites with the open reading frame that begins with the initiation codon (ATG) within the Nco I site).

2. **Purify** the insert using any standard method. We recommend using the NucleoSpin Gel and PCR Clean-Up (Cat. No.740609.50). This kit can be used for PCR clean-up or gel purification.
3. **Ligate** the digested vector and the gene fragment.
4. **Transform** chemically competent or electrocompetent bacterial cells (we recommend Clontech's Stellar Electrocompetent Cells; Cat. No. 636765) with a sample of the ligation mixture and plate on LB agar plates containing 100 µg/ml ampicillin.
5. **Identify** the desired recombinant plasmid by colony PCR, restriction analysis of miniprepped DNA, and/or sequencing of miniprepped DNA. For colony PCR, we recommend using High Fidelity PCR EcoDry Premix (Cat. No. 639280).
6. **Transform** into BL21 DE3 or other strain containing DE3 for expression analysis (see Section VIII).

VII. In-Fusion Cloning Protocol

Protocol

A. PROTOCOL: PCR Cloning of Your PCR Insert into the pEcoli Linear Expression Vectors

The following method describes a simple and highly efficient method to clone your gene in parallel into the pEcoli vectors. The magic of In-Fusion means:

- One-step directional cloning (Figure 5)
- Seamless cloning—no addition of unwanted base pairs
- No restriction digestion, phosphatase treatment, or ligation
- Clone the same PCR fragment in parallel into both expression vectors

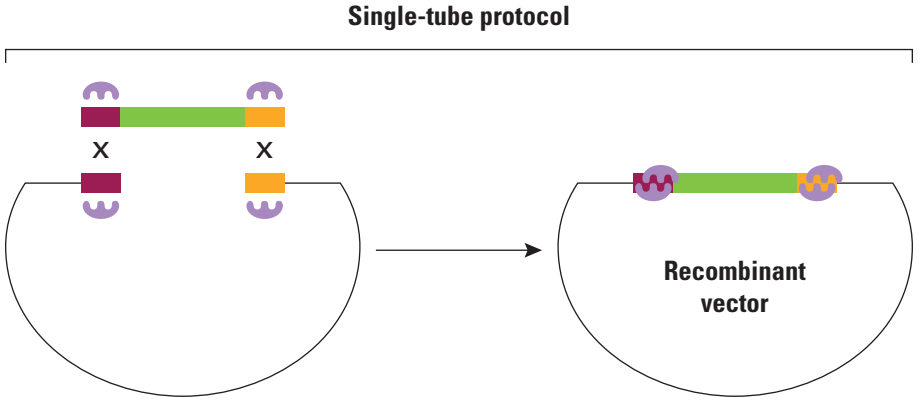

Figure 5. Simple, one-step cloning with In-Fusion PCR Cloning Kits.

1. **Design** your primers with a 15 bp homology. Visit **http://bioinfo.clontech.com/infusion/** Select "In-Fusion Primer Design Tool". Follow the on-screen directions to design your primer.

2. **Amplify** your PCR insert by using a high-fidelity enzyme such as **Advantage HD Polymerase Mix** (Cat. No. 639241).

3. **Purify** the PCR product using **NucleoSpin Gel and PCR Clean-Up** (Cat. No 740609.50). With this kit you can gel-purify or spin-column purify—the choice is yours.

   Or

   Treat with **Cloning Enhancer** (Cat. No. 639613). Cloning Enhancer removes background plasmid DNA and PCR residue, eliminating the need for PCR insert purification.

4. **Clone.** Combine your PCR insert and linearized vectors with the In-Fusion Enzyme and incubate. (The same PCR insert can be cloned into both C-term and N-term vectors).

5. **Transform and screen** colonies for the correct insert.

   See the In-Fusion Dry-Down PCR Cloning Kit User Manual (PT3754-1) at **www.clontech.com** for additional details regarding the PCR cloning procedure.

VIII. Protein Expression Protocol

A. General Considerations

Once the desired expression plasmid has been obtained, you may induce expression of your protein using IPTG, according to the following recommendations:

- First, the plasmid must be transformed into *E. coli* strain BL21 DE3 (EMD Biosciences, Inc.), which contains the T7 polymerase gene under the control of the *lac* repressor.
- It is recommended to use freshly transformed bacteria or frozen glycerol stocks for expression experiments, since continued culture or long periods of storage on plates can result in loss of expression.

The following protocols are provided for the analysis of protein expression, including protein solubility. This small-scale approach is also useful for optimizing expression conditions. The expression protocol can be scaled up for production, and IMAC purification can be performed using either **HisTALON Co resin for the highest purity** or **His60 Ni Superflow resin for the highest binding capacity**.

Protocol

B. PROTOCOL: Expression Protocol (Small-Scale)

1. Pick colonies to innoculate small (2–4 ml) LB/amp cultures and shake overnight at 37°C (ampicillin concentration is 100 µg/ml).

   In the morning, innoculate a 4–50 ml culture by diluting the overnight culture 1/20 to 1/50 with LB amp.

2. Shake until an OD of 0.6–0.8 is reached, set aside a small volume of culture (~1 ml) on ice to serve as an uninduced control, and then add IPTG to a concentration of 1 mM. Continue shaking at 37°C for 4–5 hr.
3. Centrifuge samples at 1,000–3,000 x g for 15 min at 4°C, remove supernatant, and freeze pellets at –80°C for further analysis.

Protocol

C. PROTOCOL: Analysis of Expression

You have the option to analyze total protein expression (when analyzing a number of samples), or proceed directly to analyzing solubility (see Section D). Protein solubility is essential when planning the purification protocol, since some eukaryotic proteins are insoluble when expressed in bacteria and form inclusion bodies. The trade-off of insolubility is that these proteins tend to be expressed at high levels, and isolation of inclusion bodies is an effective initial purification step:

Total expression can be analyzed by SDS-PAGE as follows:

1. Resuspend pellets in 1/10th of the original culture volume using PBS, add an equal volume of 2X SDS loading buffer (see Section III), and mix well. (It is OK to mix samples on a vortex mixer, but avoid forming bubbles if you intend to sonicate the samples.)
2. The samples can then be sonicated for 30 sec, or drawn through a 27 gauge needle 4 or 5 times. This will reduce the viscosity of the sample due to genomic DNA and thus facilitate gel loading. An alternative is to centrifuge the sample for 5 min just prior to loading the gel and use the supernatant, taking care to avoid the pellet when pipetting.
3. Heat the sample to 95°C for 3–5 minutes, centrifuge briefly, and proceed to load the gel. Samples may be stored at -20°C.

VIII. Protein Expression Protocol continued

D. PROTOCOL: Analysis of Solubility

There are numerous successful protocols for improving solubility and refolding proteins from inclusion bodies. In the latter case, the protein must be solubilized using denaturants prior to purification. IMAC purification using TALON resin works under both native and denaturing conditions. This protocol will allow you to separate soluble and insoluble (inclusion body) fractions and then analyze these by SDS-PAGE.

1. Resuspend the cell pellets in TALON xTractor Buffer:
   a. Add 20 µl TALON xTractor Buffer (see Section III) per mg of cell pellet or 40 µl per ml of the original culture volume.
   b. Incubate at room temperature for 20–30 min (the incubation with TALON xTractor buffer can be carried out on ice for 45–60 min).

NOTE: Add DNAse to TALON xTractor Buffer if the resulting sample is very viscous.

2. Alternatively, resuspend the cell pellets in 1X Equilibration/Wash Buffer as follows:
   a. Resuspend the cell pellets in a volume of the provided 1X Equilibration/Wash Buffer equivalent to 1/10th the original culture volume (chilled to 4°C).
   b. Add lysozyme to a concentration of 0.75 mg/ml. To reduce the chance of introducing proteases, use the highest purity lysozyme available.
   c. Sonicate your sample 3 x 10 sec, with a 30 sec pause on ice between each burst.
3. Centrifuge the cell extract at 10,000–12,000 x g for 20 min at 4°C to pellet insoluble material.
4. Carefully transfer the supernatant to a clean tube without disturbing the pellet. This is the clarified sample.
5. Reserve a portion for SDS/PAGE analysis. The remainder may be kept for a trial purification using TALON resin. Keep the remainder on ice until it is used for purification (carry out the purification within 2–4 hr after the extraction).
6. Resuspend the pellet in a volume of 1X Equilibration/Wash Buffer equivalent to the volume used in Step 1. Add an equal volume of 2X SDS sample buffer.
7. The resuspended pellet can then be sonicated for 30 sec, or drawn through a 27 gauge needle 4 or 5 times. This will reduce the viscosity of the sample due to genomic DNA, and thus facilitate gel loading. An alternative is to centrifuge the sample for 5 min just prior to loading the gel, and take care to avoid the pellet when pipetting.
8. Heat the samples corresponding to both the soluble and insoluble fractions at 95°C for 3–5 min and centrifuge briefly.
9. Analyze samples by SDS-PAGE. Add to this an equivalent volume of 2X SDS Sample Buffer. Typically, a sample equivalent to 25–50 µl of culture volume will produce clean bands on a gel. This corresponds to 5–10 µl of sample when processed according to the above instructions.
10. If you wish to identify the target protein band, use our Universal HIS Western Blot Kit (Cat. No. 635633) after transferring the bands to a PVDF membrane.

IX. Troubleshooting Guide

Table I. Troubleshooting Guide for Protein Expression

Description of Problem	Possible Explanation	Solution
A. Transformation of BL21 DE3 cells yields no colonies	Protein is toxic to cells	Try BL21 DE3 pLysS or pLysE cells. These strains contain plasmids which express T7 lysozyme, an inhibitor of T7 polymerase, thus raising the threshold of T7 polymerase required for expression of the gene of interest.
B. Protein is not present in cells	Incorrect cells used for transformation	Use BL21 DE3 cells or other cells which express T7 polymerase under a *lac*-regulated promoter.
	Clones contain incorrect insert	Pick another transformant.
	Protein is toxic to cells	Try BL21 DE3 pLysS or pLysE cells. These strains contain plasmids which express T7 lysozyme, an inhibitor of T7 polymerase, thus raising the threshold of T7 polymerase required for expression of the gene of interest.
	Protein may require longer induction time	Increase culture incubation time. The incubation may be performed overnight.
	Protein is degraded	Try adding protease inhibitors.
C. Protein is insoluble		Confirm presence of protein by lysing cell pellet using SDS-PAGE loading buffer and rescue solubility by altering expression conditions or expressing the protein within inclusion bodies. • **Alter expression conditions to improve solubility by promoting correct protein folding.** 1. Reduce the induction temperature to slow expression and allow time for proper folding. Try a few temperatures between 15°C and 30°C. 2. Refold protein in the presence of chaperones by transforming plasmids that express a variety of chaperones [eg., Takara Chaperone Plasmid Set (Cat. No. 3340)], or by expressing your protein in derivatives of BL21 DE3 cells that express chaperones. • **Express the protein in insoluble form within inclusion bodies, then denature and refold.** Since insoluble proteins will typically be expressed at high levels, isolation of inclusion bodies containing your protein of interest with one of the following two methods is an excellent purification step: 1. Denature the proteins within the inclusion bodies in a solution of 8 M urea or 6 M guanidine hydrochloride, then perform a gradual dilution of the denatured protein solution using stepwise dialysis. Since IMAC purification works under denaturing conditions, one has the option to purify either before or following refolding. 2. Use a commercially available refolding kit (eg. Takara Refolding CA Kits, Cat. Nos. 7350 & 7351).

X. References

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